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THE SERIAL CULTIVATION OF HUMAN DIPLOID CELL STRAINS[1]

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Only limited success has been obtained in developing strains of human cells that can be cultivated for long periods of time *in vitro* and that still preserve the diploid chromosomal configuration [41, 47, 48, 58, 59]. Indeed, heteroploidy may be a necessary corollary or even the cause of the alteration of primary or diploid cells *in vitro* to the status of a cell line. Such changes in chromosome number appear to be independent of the type of primary tissue since they have been observed in cells derived from both normal and malignant tissue [4, 22, 23, 31].

These cell lines, of which over two hundred have been reported in the literature, have serious limitations for many kinds of biological studies. Chief among these is the exclusion of their use for the production of human virus vaccines. This limitation is based on the supposition that such heteroploid cell lines, whether of normal or malignant origin, share many of the properties of malignant cells [29, 30, 37]. This objection would be even more important if viruses played a role in human neoplasia. In general, if strains of human cells could be kept continuously under conditions of rapid growth for extended periods of time with the retention of the diploid configuration these objections would not apply.

Furthermore, diploid cell strains would parallel more closely the biology of cells *in vivo*. Although characterizations of heteroploid cell lines are often stated in terms of a modal chromosomal number, this should not obscure the fact that extensive pleiomorphism is present [21]. The cells comprising the modal class in heteroploid cell lines are found to be heterogeneous if chromosomal analysis is extended beyond a simple enumeration [49]. This genomic variability constitutes an important consideration in experiments using cell lines for the study of metabolic or other phenotypic cell markers. The use of cloning as a means of reducing this variability in heteroploid cell lines is unfortunately limited by the rapid re-emergence of a range of chromosomal types among the progeny of the clone [5, 49, 50].

[1] This investigation was supported by a research grant (C-4534) from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

The results to be presented stress, incidentally, the need for a clarification of certain terms used by tissue culturists to describe a number of phenomena. Consideration of eleven important criteria show the term "cell line" to be inapplicable to the type of cells described in this report. Precedence and usage confine the term "cell line" to only those cells that have been grown *in vitro* for extended periods of time (years). This period of time presumes potential "immortality" of the cells when serially cultivated *in vitro*. (The situation is analogous to transplantable tumors which are also apparently "immortal" in the sense that serial subcultivation in proper hosts guarantees the growth of the tumor for an indefinite period of time.) The diploid cell strains presently described are assumed to lack this characteristic of potential immortality. In addition, all mammalian cell lines examined to date vary from the diploid chromosome number. This fact alone should exclude the diploid cells from being termed "cell lines" and we have chosen to refer to them as "cell strains".

A *cell strain*, therefore, is a population of cells derived from animal tissue, subcultivated more than once *in vitro*, and lacking the property of indefinite serial passage while preserving the chromosomal karyotype characterizing the tissue of origin. Conversely, a *cell line* is a population of cells derived from animal tissue and grown *in vitro* by serial subcultivations for indefinite periods of time with a departure from the chromosome number characterizing its source.

It is also possible, when observed, to include in the definition of a *cell line* the characteristic of cell "alteration" as described by Parker, Castor and McCulloch [44], and by Hayflick [17]. No such alterations have been found in the cell strains which are the subject of this report. It is proposed that the term "cell transformation", which has been used interchangeably with "cell alteration" [23], be excluded on grounds that the latter term has precedence and also that "transformation" has specific implications in the allied field of bacteriology which do not, as yet, apply to cell culture. The terms "established cell lines" and "stable cell lines" should likewise be avoided as the former is redundant and the latter implies that changes have ceased to take place or are no longer possible. The term *primary cells* should indicate those cells obtained from the original tissue that have been cultivated *in vitro* for the first time. If subsequent passages of these cells are made, it is assumed that such cells can properly be called a *cell strain* until they are either lost through further subcultivations or alter to the heteroploid state, in which case they could properly be referred to as a *cell line* (Text-Fig. 1).

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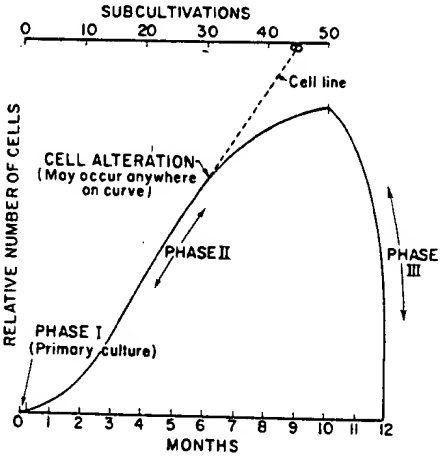
of 25 strains of human cells derived from fetal tissue which retain true diploidy for extended periods of cultivation without alteration. Specific attention will be given to those cell characteristics which serve to distinguish cell lines from cell strains.



Text-Fig. 1.—Diagrammatic representation of the history of cell strains and the phenomenon of cell alteration. Phase I, or the primary culture, terminates with the formation of the first confluent sheet. Phase II is characterized by luxuriant growth necessitating many subcultivations. Cells in this phase are termed "cell strains". An alteration may occur at any time giving rise to a "cell line" whose potential life is infinite. Conversely, cell strains characteristically enter Phase III and are lost after a finite period of time.

MATERIALS AND METHODS

Media.—The growth medium (GM) used was Eagle's Medium in Earle's Balanced Salt Solution [8] supplemented with 10 per cent calf serum.[1] Twenty-five ml of 5.6 per cent $NaHCO_3$, 10^5 units of penicillin and 10^5 μg of streptomycin, were added per liter. The final pH of the medium was 7.3, and before use it was brought to 37°C. Phosphate buffered saline (PBS) was prepared as described by Dulbecco and Vogt [7]. Difco trypsin (1:250) was prepared as a 0.25 per cent solution in PBS and supplemented after filtration with the antibiotics described above.

Isolation of primary cells.—Two methods of cell cultivation from primary tissue were employed in this study with identical qualitative results. The use of trypsin yielded far more cells initially than cultures prepared from fragmented or minced tissue. Since high cell yields were not required from the starting tissue, most cultures were started from fragmented or minced tissue. Such preparations gave fewer cells initially than could have been obtained from tissue treated with the enzyme preparation. Minced preparations were obtained by cutting the tissue in a Petri dish containing GM with paired scalpels or a scissors until the size of each piece approximated 1–4 mm^3. Fragmented preparations were obtained by tearing apart the tissue with two pairs of forceps in a Petri dish containing GM until the pieces could no longer conveniently be grasped and shredded. The entire contents of the dish were emptied into one or more Pyrex Blake bottles (surface area 100 cm^2), depending on the size of the original starting tissue. The fragmented lungs, for example, from a three-month-old human fetus were usually placed in four Blake bottles. Treatment of tissue with trypsin was done, in general, according to the method of Fernandes [11].

[1] Obtained from Microbiological Associates, Inc., Bethesda, Maryland.

Initiation of cultures.—If the fetal tissue was viable when received, cells could be found in bottles planted by any one of the methods described after about three days of incubation at 36°C. When growth was first observed the cultures were refed. The spent medium and any fragments present were discarded. If additional bottles were required these fragments could be replanted in a new bottle. Fresh GM was added and as soon as the cells formed a confluent sheet the cultures were subcultivated. This normally occurred in about 10 days. Periodic feeding of the cultures was done when a sharp drop in the pH of the medium made it necessary.

In the beginning of this study attempts were made to minimize the period of time elapsing between the receipt of the fetus or fetal tissue and its cultivation *in vitro*. It was subsequently found that if either was viable upon receipt it could be kept for at least 5 days at room temperature, or 5°C, without apparent loss of viability. Minced tissue, kept in a minimal amount of GM has been found to be viable for periods of time up to 3 weeks, either at room temperature or 5°C.

Subcultivation of confluent cultures.—As soon as cell cultures were fully sheeted they were put on a strict schedule of subcultivations, which were done alternately every third and fourth day. The spent GM was discarded and trypsin solution was added to each bottle. After incubation at 37°C, or room temperature, for 15 min, the enzyme solution containing the dislodged cells was centrifuged for 10 min at 600 r.p.m. in an International Size 2 Model V Centrifuge. The trypsin solution was decanted after centrifugation and the cells were resuspended in a small amount of GM, aspirated with a 5 ml pipette, and evenly distributed to two Blake bottles. Sufficient fresh medium was added to each bottle to cover the surface adequately. This was called a 2:1 split. In the early part of this study split ratios of 3:1 were used with equal success. Incubation was carried out at 36°C.

Preservation of cells by freezing.—After trypsinization of a mature culture and resuspension of the centrifuged cells in a few ml of GM, the cell concentration was adjusted with GM to 1.5–2.0 $\times 10^6$ cells per ml. Sterile glycerol was added to give a final concentration of 10 per cent and the suspension was dispensed in 2 ml portions in 5 ml ampules. The ampules were then sealed and held at 5°C overnight. The next day the ampules were placed directly at −70°C.

Recovery of frozen cells.—Ampules to be reconstituted were removed from the dry ice chest and placed quickly in a 37°C water bath. After the contents had thawed, the suspension was placed in a milk dilution bottle (surface area 40 cm^2) and sufficient fresh GM added to cover the surface of the bottle adequately. After incubation at 36°C for one day the medium was completely changed. Periodic feedings of the culture were made until the cell sheet was confluent at which time the culture was manipulated as described above. Reconstituted cells frozen for up to one year invariably yielded viable cultures if these conditions were met. Although quantitative recovery of the frozen cells was not achieved, the fraction of the frozen population that did survive was always sufficient to recover the culture.

Chromosome analysis.—Thirteen of the strains were studied for purposes of chromosome analysis. Actively dividing cultures (usually 48 hr after seeding a Blake bottle) of these strains were sacrificed for chromosome studies of cells arrested in metaphase by colchicine treatment. Following 6 hr subjection to a concentration of 2×10^{-6} *M* colchicine in the medium, the cells were trypsinized free. Suspended cells were then processed for spreading on glass slides according to an air-drying technique [38]

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based upon that of Rothfels and Siminovitch [51]. Aceto-orcein stained chromosome preparations were scanned under low power optics (20 × objective) for adequately spread metaphase cells showing a minimum of scattering of the chromosomes. Selected metaphases were then studied under oil immersion optics for simple counts and for detailed analysis of karyotype, where warranted.

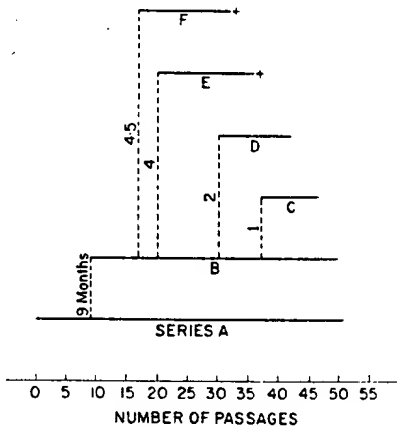
From 250 metaphases per determination the proportions of polyploid and diploid cell classes were obtained by rough chromosome estimates (approximate accuracy ±6 chromosomes).

Sex chromatin.—The scoring of nuclei for the presence or absence of the sex chromatin, or Barr body [3], was done upon the same slide preparations made for the study of hypotonically spread metaphases. Interphase nuclei which were not overstained and which did not have numerous chromocentres were scored for sex chromatin determinations. These considerations required the elimination of 30–40 per cent of the interphase nuclei from the scoring. This ambiguous class of nuclei was considerably reduced by using less intense staining with aceto-orcein as follows.

Coverslips bearing sheets of fibroblasts were rinsed briefly in PBS, fixed in methyl alcohol for 1 min, air-dried, aceto-orcein stained for 10–30 sec, rinsed twice briefly in each of 95 per cent ethyl alcohol, absolute ethyl alcohol, xylol, and then mounted in Permount.

These provided excellent detail of the sex chromatin body with very few overstained nuclei. Only those nuclei which formed numerous chromocentres, especially common in non-dividing cultures, comprised the ambiguous class, while 80–90 per cent of the remaining female nuclei clearly displayed a single sex chromatin body. In male material lightly stained in this way 80–90 per cent of the nuclei (excluding those with many chromocentres) clearly lacked any such stained body. A minimum of 200 nuclei were scored per determination.

Implantation of diploid cells into hamster cheek pouches.—Cells of diploid strain WI-25 at the 14th passage (61 days *in vitro*) were selected for inoculation into the cheek pouches of unconditioned hamsters. In general, the technique used was that described by Handler and Foley [12, 15]. Trypsin dispersed cells were centrifuged and resuspended so that the desired inoculum was contained in 0.1 ml of GM. Inoculum sizes ranged from 10^3 to 10^3 cells per 0.1 ml. Five hamsters were inoculated with



Text-Fig. 2.—Diagrammatic representation of the history of the WI-1 strain of human diploid cells. Series A represents the continuous subcultivation of the strain through a period of 50 passages during which time surplus cultures from each passage were committed to storage at −70°C. Other series denote those cultures of the strain that were removed from storage at −70°C after periods of time represented by the vertical dash line. Surplus cultures from each series were committed to storage, the origin of the dash line denotes that passage which was thawed to start a new series. ——, series; – – –, months; +, still in cultivation as of February 28, 1961.

each cell concentration selected. For control purposes, five hamsters were also inoculated with 10^5 HeLa cells per 0.1 ml.

Homotransplantation of WI-1 cells into terminal cancer patients.—Two pools of strain WI-1 cells were used to inoculate six terminal cancer patients. The first pool consisted of those cells that had been grown serially for nine passages, held for 9 months at −70°C, restored, carried for an additional 21 passages, stored again at −70°C for 2 months, restored and subsequently carried for another seven passages (Series D in Text-Fig. 2). Thus this first pool of cells represented a total of 37 subcultivations. The second pool of WI-1 cells consisted of cells that had been grown serially for nine passages, held for 9 months at −70°C, restored, and subsequently carried for 36 more passages (Series B in Text-Fig. 2). Thus the second pool of cells represented a total of 45 serial subcultivations *in vitro*.

The cells of both pools were grown as indicated previously, harvested with trypsin, resuspended in PBS and adjusted to a concentration of 6×10^5 cells per ml. Cell counts were made in trypan blue and only viable cells were counted; dead cells constituted less than 3 per cent of the total harvest. One-half ml of this suspension was inoculated subcutaneously on the flexor surface of the forearm with a tuberculin syringe fitted with a No. 20 needle. The area was tattooed for subsequent identification when taking biopsies. The six patients used in this study were also skin tested with GM alone prior to cell inoculation in order to ascertain their sensitivity to calf protein. These patients had advanced incurable cancer and a very short life expectancy.

EXPERIMENTAL RESULTS

Establishment of diploid cultures.—In all cases where the original human fetal tissue was viable, indicated by cell growth in the first culture, the strains from the various organs were kept in serial cultivation as shown in Table I. When a particular fetus was found to yield non-viable cells from one organ, invariably cultures made from other organs were also found to be non-viable. It was found, therefore, that if a primary culture was obtained from tissue, the cell strain could be cultivated serially for periods of time up to 11 months. With the exception of strains WI-6 and WI-22 derived from heart tissue, all strains could be carried for at least 25 passages, extending over a period of 5 months. The maximum number of subcultivations obtained is exemplified by the WI-23 strain derived from lung, which lasted for 8 months during which 55 subcultivations were made. Without exception, all of the human strains were of the fibroblast cell type from about the 5th subcultivation. The kidney strains began as epithelial cell cultures with scattered nests of fibroblasts. The least successful cultures were those obtained from liver in which fully sheeted primary cultures were rarely obtained.

Although the subject of this report is confined to experiments involving human fetal cells, adult human cells have also been carried for similarly

extensive periods of time with retention of the diploid configuration. Other workers [41, 59] have reported similar results with adult human diploid cells.

Morphology of diploid human fibroblast cell strains.—Figs. 1 and 2 represent fibroblasts of the WI-1 strain of diploid human fetal lung cells after 35 subcultivations and 9 months *in vitro*. Characteristically these cells are extremely elongated fibroblasts averaging about 185 $\mu \times 15$ μ. The single nucleus contains from 1 to 4 nucleoli which vary from oval to branching bodies. Individual cells are markedly transparent with characteristically

TABLE I. *History of human diploid cell strains.*

Strain designation	Fetus no.	Tissue of origin	Months in serial cultivation[a]	No. of subcultivations
WI-1	1	Lung	11	51
WI-2		Skin and muscle	6[b]	20[b]
WI-3	2	Lung	5	35
WI-4	3	Kidney	6	29
WI-5		Muscle	7	33
WI-6	4	Heart	2.5	10
WI-7		Thymus and thyroid	5	25
WI-8	5	Skin	8.5	32
WI-9		Kidney	8.5	29
WI-10	6	Kidney	5[c]	32[c]
WI-11	7	Lung	5[c]	30[c]
WI-12	8	Skin and muscle	8	41
WI-13		Kidney	8	40
WI-14	9	Skin	8	43
WI-15	10	Kidney	7.5	28
WI-16	11	Lung	8	44
WI-17		Liver	5[b]	24[b]
WI-18	12	Lung	8	53
WI-19	13	Lung	8	50
WI-20	14	Skin and muscle	5[b]	25[b]
WI-21	15	Heart	5	26
WI-22	16	Heart	1	5
WI-23	17	Lung	8	55
WI-24	18	Lung	7	39
WI-25	19	Lung	6[c]	38[c]

[a] Continuously passaged cells, never reconstituted from frozen stock (Series A).
[b] Serial cultivation of strain lost through bacterial contamination but cells from previous passages stored at −70°C.
[c] Still in culture as of February 28, 1961.

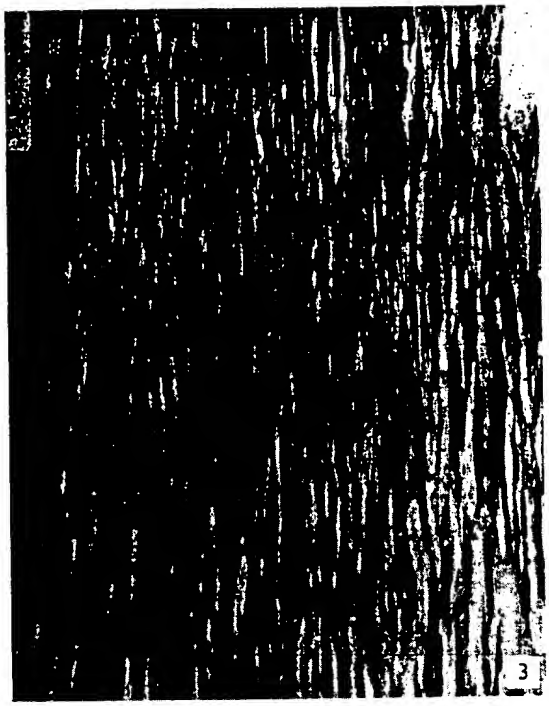


Fig. 1.—Strain WI-1. Diploid human fetal lung cells after 35 subcultivations and 9 months *in vitro*. Phase contrast. ×360.

Fig. 2.—Strain WI-1. Diploid human fetal lung cells after 35 subcultivations and 9 months *in vitro*. Stained with May-Grünwald Giemsa. ×300.

fine cytoplasmic granularity. The cell membrane is filamentous at either end of the long axis of the cell and undulating membranes are common. Multinucleated cells are rarely found. The cells adhere firmly to the glass surface about 1 hr after subcultivation. Within 48 hr many highly polarized fibroblasts oriented in different directions can be seen. At this time, under ideal conditions of growth, almost every field (150 ×) contains from two to eight rounded, highly refractile cells which represent fibroblasts in various stages of mitosis. As the cell sheet becomes confluent the sheet takes on a quilt-like appearance when the bottle is examined macroscopically with incident light. Upon microscopic examination, the sheet is seen to be composed of numerous swirls of fibroblasts, each swirl containing elongated cells oriented in a common direction (Fig. 3). The direction of cell orientation resulting from parallel alignment varies from area to area. It is this growth pattern that gives to the bottle its characteristic "sheen" when the cell sheet is viewed with incident light macroscopically. Puck [46] has described the appearance of such fibroblasts, growing in Petri dishes for studies of plating efficiency (Fig. 4), as "rough, hairy, colonies". The concept of contact inhibition as described by Abercrombie and Heaysman [1] seems to explain adequately this growth pattern. When the movement of a fibroblast in a particular direction brings it into contact with another fibroblast so that an adhesion forms, further movement in that direction is inhibited. All these characteristics apply to each of the human diploid cell strains throughout their periods of active proliferation.

Chromosome analysis.—To determine if heteroploid or aneuploid classes had emerged within the growing population of these strains the level of tetraploidy was determined, exact chromosome counts made and karyotypic analyses of the better metaphases prepared (Table II). Exact chromosome counts of 28 or more metaphases per determination revealed primarily diploid and few tetraploid cells[1] (Table II). The level of tetraploidy at various passages was found to be low, exceeding 3 per cent in the dividing population only in strain WI-25 (Table II). There was no evidence of alteration in number or morphology of the somatic cell chromosomal comple-

[1] Exceptions with respect to available metaphases for exact counts being strains WI-15 and WI-1 of the 39th passage in which mitoses were scanty (Table II).

Fig. 3.—Strain WI-1. Directional orientation of cells after 3 days in culture. Stained with May-Grünwald Giemsa. ×100.

Fig. 4.—Strain WI-1. Colony development from single cells planted in a 50 mm Petri dish. Photographed after 35 days in culture. Colonies are rough and hairy in appearance. May-Grünwald Giemsa.

ment. Karyotypic analyses of 6–12 excellent metaphases for each determination revealed all 23 chromosome pairs present and recognizable either individually or according to their group: #1, #2, #3, #4-5, X-6-12, #13-15, #16, #17, #18, #19-20, #21-22-Y as defined by the Denver Conference of 1960.[1, 2]

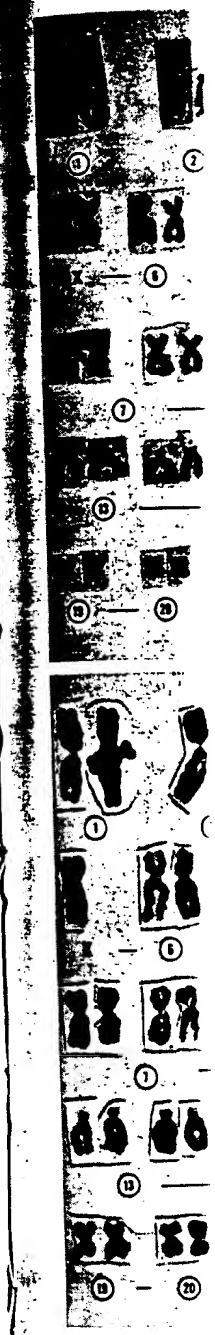
TABLE II. *Karyology of human cell strains.*

Strain	Passage no.	Chromosome counts (exact counts only) 40	41	42	43	44	45	46	47	48	91	92	Other counts	Tetraploids per 250 metaphases	Sex chromosomes
WI-1 (Ser. A)	9						1	56						3 (1.2 %)	XY
WI-1 (Ser. B)	21[a]			1	—	1	1	24			1	1	59 ± 0 88 ± 0	4 (1.6 %)	XY
WI-1 (Ser. D)	39 (Phase III)			1	1	—	—	14					38 ± 0	4	XY
WI-12	32	1	1	—	1	—	3	26						2 (0.8 %)	XX
WI-12	40					1	1	31						2	XX
WI-13	25						1	25						3	XX
WI-14	29				1	1	7	25	1	2	—	1	57 ± 0 54 ± 0	3	XY
WI-15	21				1	—	—	7						—	XY
WI-16	22				1	1	4	25			—	1		3	XX
WI-17	17				1	1	2	27						3	XX
WI-18	21				2	1	1	26					40 ± 0 52 ± 0	6 (2.4 %)	XY
WI-19	21		1		1	2	—	24						2	XY
WI-21	17					1	4	26						1	XY
WI-23	22			1	—	1	2	25						3	XY
WI 24	22			1	—	—	2	27			—	1		4	XX
WI-25	30		1		1	—	1	24			—	1	68 ± 0 62 ± 0	16 (6.4 %)	XX

[a] Period of 9 months in frozen state at 9th passage. See Text-Fig. 2.

Those counts falling below the 2*n* number of 46 (Table II) are regarded as the result of excessive spreading and occasional loss of one or a few chromosomes from a complement. This is supported by the general "left-skewness" of such counts and the lack of consistency with respect to the identity of the

[1] Representative karyotypes of male and female diploid strains are shown in Figs. 5–8.
[2] Report of a study group: *Am. J. Human Genet.* **12**, 384 (1960).



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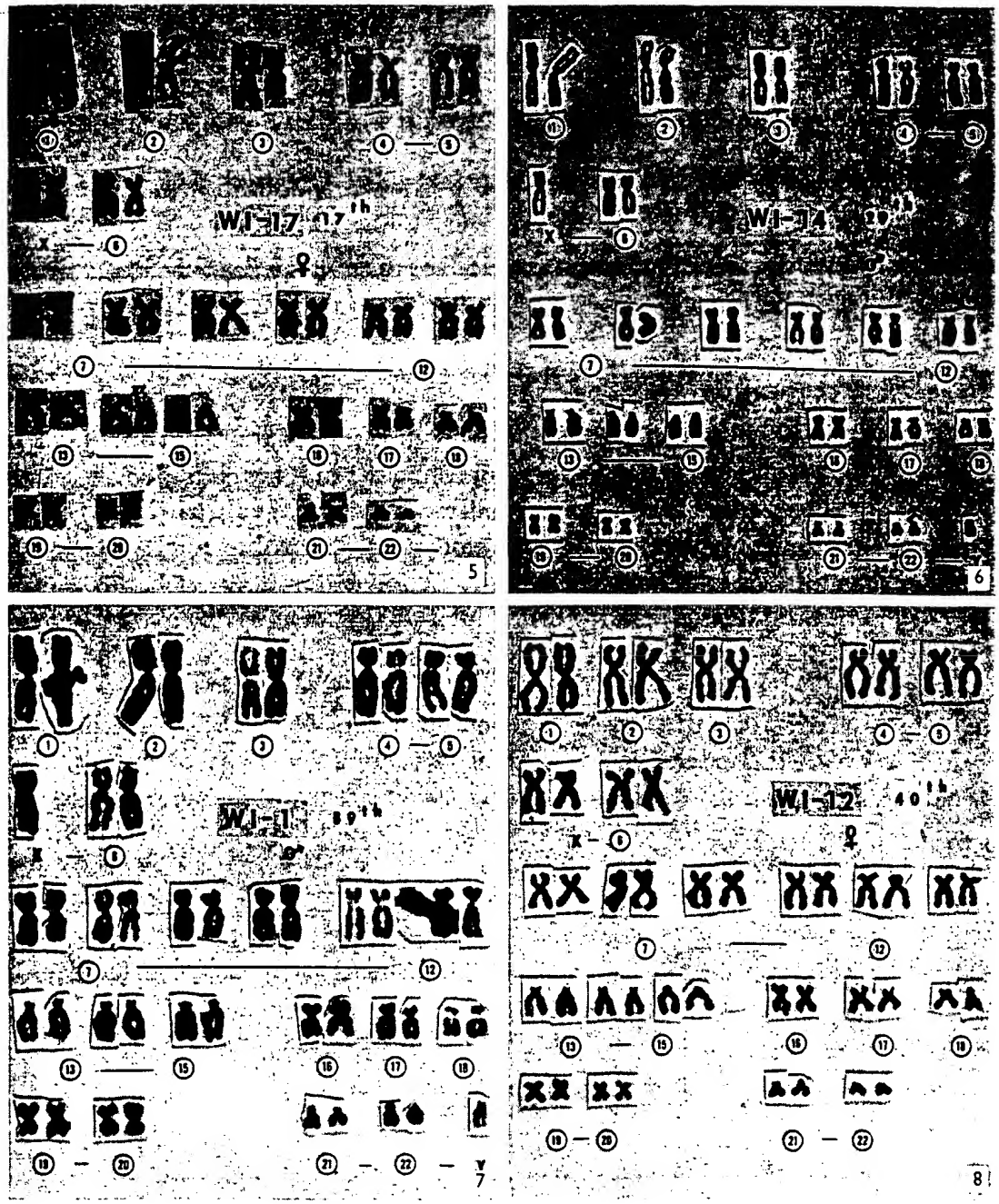

Fig. 5.—Strain WI-17, 17th passage. Classic female diploid karyotype of metaphase cell.

Fig. 6.—Strain WI-14, 29th passage. Classic male diploid karyotype of metaphase cell.

Fig. 7.—Strain WI-1, 39th passage. Classic male diploid karyotype of metaphase cell. Note that one member of pair #1 overlies a member of pair #11.

Fig. 8.—Strain WI-12, 40th passage. Classic female diploid karyotype of metaphase cell.

missing chromosome in each case. Approximately half of these seeming aneuploid counts were analyzable and in only one case (WI-14) was the missing chromosome from the same group. Thus four of seven counts at $2n-1$ lacked a chromosome from group X–12. The usually rare $2n+1$ and $2n+2$ counts here might be regarded as further evidence for suspicion of this strain. On the other hand, this particular material was excessively spread and broken metaphase groups were common.

Sex chromatin.—The retention of a single sex chromatin body in the interphase nucleus was noted in those strains having a female (XX) karyotype throughout all passages examined (Table II). Each of the four female strains was readily detected from a coded group of slide preparations which included seven male strain representatives. Since this material was prepared primarily for chromosome studies, 30–40 per cent of the nuclei were not included in the scoring. Of the suitable nuclei remaining 57–66 per cent had a single chromatin mass (Figs. 9 and 10), usually peripheral, such as described by Barr [3]. In male material 10–32 per cent of the scorable nuclei possessed smaller chromatic bodies which might be considered as sex chromatin. However, these bodies did not permit interpretation as typical bipartite sex chromatin. Male nuclei regarded as lacking sex chromatin are shown in Figs. 11 and 12. Presumptive tetraploid nuclei (size class) in female cells usually displayed two distinct sex chromatin bodies (Fig. 13). This finding is consistent with the extensive work of Klinger and Schwartzacher [28], who used human amnion in studies also involving spectrophotometric measurements of sex chromatin. Other examples of female strain nuclei with sex chromatin are presented in Figs. 14 and 15. Examination of female cell strains at high passages revealed no reduction in the proportion of nuclei with the typical body. (Strain WI-16 in the 44th passage showed 82 per cent with single body, 9 per cent with two bodies, less than 2 per cent with three bodies and 8 per cent with no visible body. Total: 237 nuclei.)

Fig. 9.—Strain WI-16, 22nd passage. Single sex chromatin bodies (arrows) in nuclei from female cells. (Chromosome preparation: hypotonically pretreated, 15 min in orcein.) Note dense nuclei. ×1050.

Fig. 10.—Same material as in Fig. 9. ×1050.

Fig. 11.—Strain WI-1, 9th passage. Absence of sex chromatin in a nucleus from male cells. Chromosome preparation. ×1050.

Fig. 12.—Strain WI-1, 22nd passage. Absence of sex chromatin in a nucleus from male cells. Chromosome preparation. ×1050.

Fig. 13.—Strain WI-25, 16th passage. Single sex chromatin bodies (arrows) in diploid nuclei from female cells. Note 2 sex chromatin bodies in the large presumptive tetraploid nucleus. (Coverslip grown preparation: 10 seconds orcein.) ×1000.

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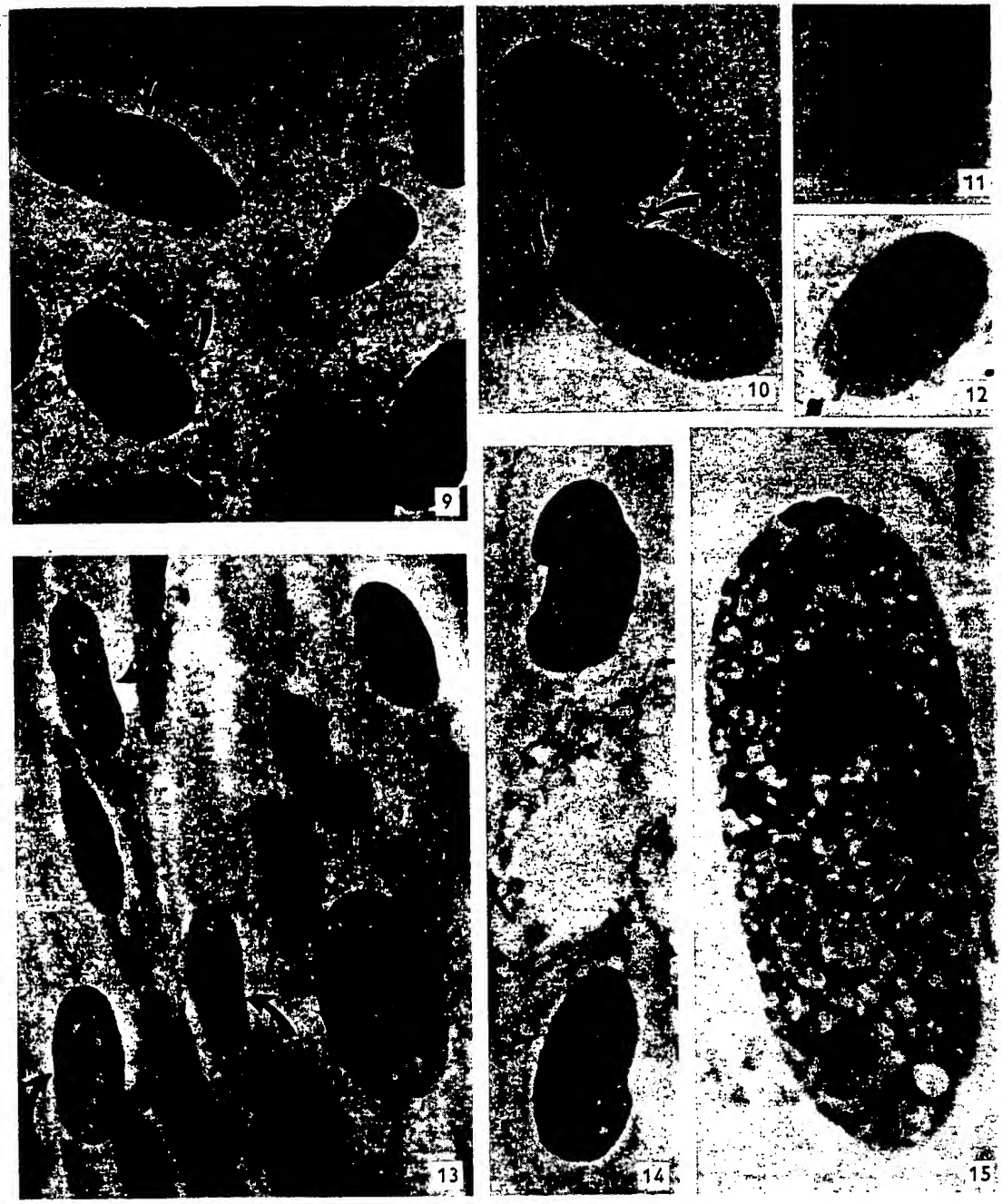

Fig. 14.—Strain WI-16, 44th passage. Single sex chromatin bodies in nuclei from female diploid cells. (Coverslip grown preparation: 10 seconds orcein.) ×1200.

Fig. 15.—Strain WI-16, 44th passage. Presumptive tetraploid nucleus with two adjacent sex chromatin bodies located peripherally. Female strain. (Coverslip grown preparation: 10 seconds orcein.) ×2800.

General growth characteristics of human diploid cell strains.—The diploid cell strains were, in all cases, established without recourse to special procedures. Once the cultures have become confluent cell sheets, the cells are very active metabolically, as shown by the fact that the GM becomes acid faster than in cultures of heteroploid cell lines inoculated with the same number of cells. In general, all diploid strains were subcultivated with a split ratio of 2:1, twice a week. This split ratio and rigid semi-weekly schedule of subcultivation was maintained until the last passage, when the cultures failed to show the numerous mitotic figures characteristic of good growth and started to accumulate debris. These degenerative changes did not appear suddenly, but took place over a period of from 1 to 3 months (Figs. 16 and 17). The fine debris appears to be the first indication that the strain is waning. The debris aggregates about the cell surfaces and presumably consists of protoplasmic material resulting from cell degeneration. In addition to the loss of mitotic activity and accumulation of debris, the cells become much less polarized, more spread out, and lose contact inhibition. Although mitotic activity eventually ceases in the culture, acid production continues; and as dictated by drops in the pH, cultures can be fed for a few months until all acid production ceases and the culture is observed to have completely degenerated.

At the first signs of these changes, various attempts were made to recover the strain. Changes in media components, further subcultivations and attempts to crowd into one culture a number of cultures of degenerating cells all failed to accomplish the intended result. It is apparent, however, that media composition plays no role in explaining the cause of this degeneration, since portions of the same large pool of medium can be used to support the luxuriant growth of "young" strains while older strains are observed to degenerate in the same pool of medium. Even strains derived from different organs of the same fetus support this contention. A lung strain may be degenerating while a skin strain is luxuriating in the same pool of medium (which is prepared in large enough batches to last a number of weeks). Even cells which are

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Fig. 16.—Strain WI-1, 50th passage. Degenerative changes characteristic of Phase III. Mitosis ceases and granular debris accumulates. Unstained. ×140.

Fig. 17.—Same as Fig. 16.

Fig. 18.—Strain WI-1, 30th passage. Degeneration of cells in the presence of Poliovirus Strain Koprowski-Chat Type 1 (attenuated virus). ×140.

Fig. 19.—Strain WI-1, 30th passage. Plaque formation in the presence of Poliovirus Strain Koprowski-Chat Type 1. 50 mm Petri dish.


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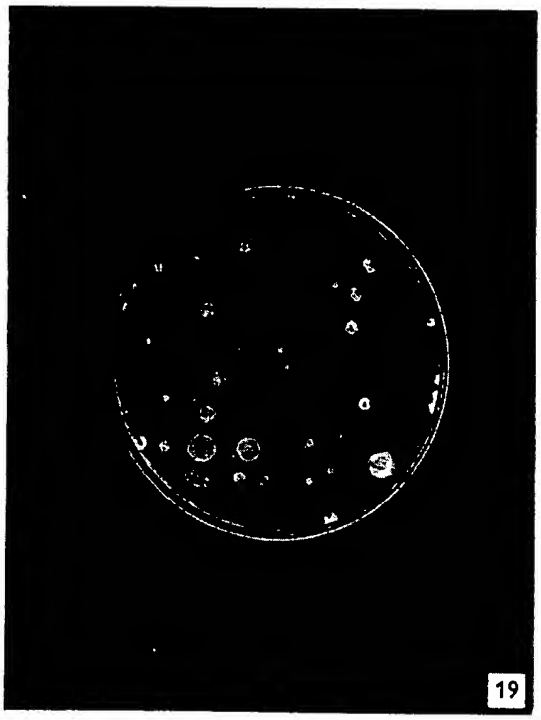
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reconstituted (e.g.) from the frozen 9th or 37th passage flourish in the same medium that was used on the identical strain undergoing degeneration in the 50th passage (Text-Fig. 2). Such observations were confirmed many times whenever a diploid strain started to degenerate.

The general history of a diploid cell strain may, therefore, be divided into three distinct phases (Text-Fig. 1). Phase I, or early growth phase, constitutes that period when the cells have been freed from the intact tissue and are just establishing themselves on glass (primary culture). In general this phase lasts from 1 to 3 weeks. Phase I ends with the formation of the first confluent sheet, at which time the culture is ready for its first subcultivation and entry into Phase II. Phase II is characterized by rapid cell multiplication and great acid production. During this phase the diploid strains must be subcultured at least twice a week with split ratios of 2 or 3:1. This coincides with the formation of confluent sheets. Apparently, success in keeping the diploid strains in serial cultivation for long periods of time depends on their being kept in the log phase of growth as much as possible during their history. Phase II lasts from 2 to 10 months, at the end of which time cell degeneration begins to take place. This degeneration and lessening of mitotic activity heralds the appearance of Phase III or the terminal phase. It is characterized by the appearance of debris, as illustrated in Figs. 16 and 17, reduction of mitotic activity and a consequently longer period of time for the development of confluent sheets. The frequency of tetraploids among the few dividing cells of this degenerative phase was as low as it was at all passage levels (Table II, WI-1 Series D). However, in interphase cells of Phase III, bizarre nuclear forms and sizes become more frequent and the appearance of these nuclei is reminiscent of irradiated cultures. Our attempts to reverse Phase III have been uniformly unsuccessful.

Absence of pleuropneumonia-like organisms (PPLO) and latent viruses in human diploid cell strains.—Periodic monitoring of all diploid cell strains herein reported has never revealed the presence of PPLO as a contaminant. The techniques used were those described previously by Hayflick and Stinebring [19]. These results were predictable on the basis of evidence recently presented describing the probable origin of this type of contaminant [18].

Numerous attempts were made to detect the presence of latent viruses in all the diploid cell strains. Spent medium with and without cells was passed into many types of tissue cultures and laboratory animals with consistently negative results. Special attention was given to spent fluids and cells from cultures in Phase III. In no case was cytopathology (or hemadsorption) observed in any of the cell strains before or during Phase III that could be attributed to

the unmasking of latent viruses, as is the case, for example, with primary monkey kidney cells [24] or primary human tonsil and adenoid tissue [52, 53]. Cells obtained from Phase II and Phase III were stained by various methods and observed for possible virus inclusions with negative results.

In order to investigate this point more critically, a culture of strain WI-1 (known to be male) at the 49th passage was selected for the following experiment. Since this strain was in Phase III it had not formed a confluent sheet but consisted of numerous scattered cells that were still lowering the pH when fed. This culture was seeded directly with a suspension of female strain WI-25 in Phase II which was then at the 13th passage; sister cultures of WI-25 were carried simultaneously. The mixed culture (WI-1 plus WI-25) began to proliferate as expected and has now been subcultured 17 times, which represents the identical number of passages of the WI-25 sister culture from the date of the experiment. In addition, cells examined at this passage of the mixed culture have been characterized as female by the presence of sex chromatin and also by karyotype analysis (Table II). Single sex chromatin body: 79 per cent; double: 10 per cent; triple: less than 1 per cent; none: 10 per cent (418 nuclei). If a latent virus had been responsible for Phase III in the WI-1 strain it seems unlikely that it would have been able to discriminate between male and female cells.

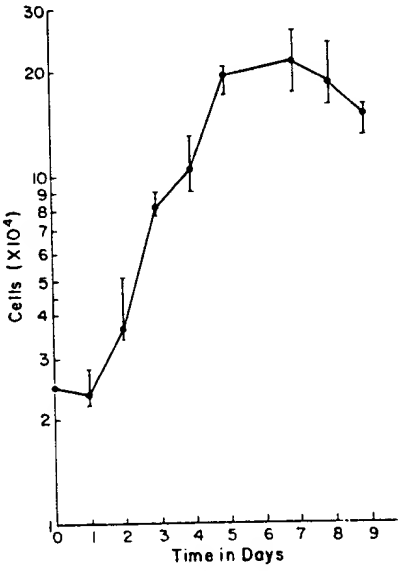
Although it cannot be said with certainty that any cell line or cell strain is absolutely free of contaminating viruses our evidence does not suggest the presence of such viruses.

Growth rate of human diploid cell strain WI-1.—WI-1 cells at the 25th subcultivation were used to determine the rate of growth. These had been in continuous cultivation for 5 months. The growth curve of these cells is shown in Text-Fig. 3 and was in general derived according to the method of Fernandes [11]. Each point is the average of four separate tube cultures, inoculated initially with 2.5×10^4 cells per ml per tube. The counts show viable cells only, ascertained by trypan blue staining. The generation time of 24 hr obtained during log phase does not differ significantly from that obtained for HeLa [20]. An initial lag of 24 hr was found, after which time the cells entered the log phase for 96 hr. After approximately 9 days the cell numbers remained constant with viability remaining at 90 per cent for one month.

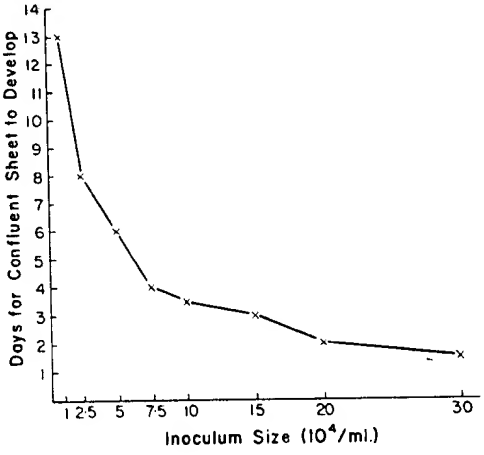
Size of initial cell concentration as a function of population survival.—Early in these studies it was found that, under the conditions described, both a minimum concentration of 10^4 cells per ml and a period of 13 days were necessary to obtain a confluent culture in bottles or tubes as indicated in

Text-Fig. 4. Cell concentrations below 10^4 per ml did not result in confluent sheet development but in isolated islands of cell colonies (Fig. 4).

It has been consistently observed that per unit area of glass surface about 25 per cent fewer diploid cells are found than cells of heteroploid cell lines (e.g. HeLa) in confluent cultures.



Text.-Fig. 3.



Text.-Fig. 4.

Text-Fig. 3.—Rate of growth of strain WI-1, 25th passage. Four separate tube cultures were used for each point on the curve. The starting inoculum was 2.5×10^4 cells per ml per tube.

Text-Fig. 4.—Period of time necessary for confluent sheet formation starting with different inoculum sizes. Strain WI-1, 27th passage.

Long-term maintenance of tube cultures.—Various media were investigated in order to determine the best medium for maintaining tube cultures of diploid cells for long periods of time. Pyrex test tubes were inoculated with 5×10^4 cells each of strain WI-25 (14th passage) in 2 ml each of the medium to be tested. The media used were GM with 1, 5, and 10 per cent calf or horse serum; medium 199 [39]; and Eagle's medium supplemented with 50 per cent bovine amniotic fluid. One set of tubes was not refed, another set was refed once a week and the third set twice a week. No pH changes were recorded for those sets that were refed. The results indicated that those cultures in which 1 per cent serum was used maintained themselves in the best condition for 4 weeks whether or not fluid changes were made. Horse serum in concentrations greater than 1 per cent sometimes caused cell granularity.

A trial was then made of the various media containing no serum. Strain WI-11 (8th passage) was used after being washed once with medium 199. Those cultures refed once a week, regardless of the medium used, survived most successfully for 4 weeks with no added serum.

In order to restrict pH reduction by tube cultures being maintained on serum-containing media, cultures of WI-11 were maintained with 1, 0.5, 0.25, and 0.1 per cent calf serum, in Eagle's Minimum Essential Medium [9] containing 0.01 *M* final concentration of tris buffer. Although the cells metabolized well without addition of tris buffer, the pH was more easily maintained in its presence. After 4 weeks with no refeeding, excellent tube cultures were obtained with all serum concentrations in tris buffered Eagle's Minimum Essential Medium. The pH had dropped from 7.8 to 7.1.

The diploid strains are therefore unusually well-suited for virus isolations where single cultures may have to remain in a viable state and metabolically active for long periods of time without fluid changes. The fact that excellent maintenance was obtained with serumless media also recommends them as important hosts for many virus studies.

Lack of growth of human diploid cell strains in suspended cultures.—Many attempts were made to grow cells from diploid human strains in suspended cultures involving cells from various passage levels. In spite of the changes made in such parameters as inoculum size, medium components, speed of agitation, and the condition of the harvested cells for suspended culture inoculation, success was never achieved. A steady decline of the viable cells inoculated was observed in every case and within three days the entire suspended culture was dead. It appears, therefore, that human diploid cell strains share with primary cells an apparent failure to multiply from individually suspended cells in agitated culture. Under conditions which have repeatedly resulted in successful suspended cultures of HeLa and WISH [17] (both of which are heteroploid cell lines), diploid human cell strains failed to grow.

Restoration of diploid human cell strains from frozen stock.—Although present evidence indicates that human diploid cell strains cannot be carried continuously for an indefinite period of time as is usual with cell lines, it is possible to have a diploid cell strain *continuously available*. This is achieved by storing diploid cells at −70°C from any or all of the excess cultures that are made when the strains are subcultured and before they enter Phase III. Thus it was possible, for example, in the case of the WI-1 strain, to have stored at −70°C ampules of cells derived from almost every passage up to the 50th, at which time the strain entered Phase III and excess cells were

unobtainable. In this case where the WI-1 cells were lost after the 51st subcultivation and 11 months in continuous cultivation, it was possible to restore from frozen stock those cells that were derived from, for example, the 9th passage of this strain. These cells had been frozen for 9 months and the culture successfully restored. The WI-1 cells restored from frozen 9th passage cells and representing Series B of this strain were cultivated for an additional 41 passages over 5 months. Excess cells derived from each of the 41 passages of Series B were also frozen at −70°C and restoration achieved. The cells from Series B entered Phase III at the 41st passage after restoration of the 9th passage cells, or a total period of 50 passages. As indicated in Text-Fig. 2, restoration of other series of WI-1 cells from frozen stock yielded cultures that, regardless of the passage frozen, entered Phase III at total cumulative passages of 42 or better.

It is apparent, therefore, that by freezing cells at each subcultivation or every few subcultivations one could have cells available at any given time and in almost limitless numbers.[1] If it is assumed that the cell strains have a "passage potential" of 50 passages, as in the WI-1 strain, almost unlimited numbers of cells can be obtained by restoring cells frozen from surplus cultures at each passage and committing the excess cultures of this second series of cells to the freezer. This pattern can presumably be repeated until the theoretical progeny limit of 10^{22} cells has been achieved (Text-Fig. 2). Diploid cell strains restored from the frozen state still retain all those characteristics investigated for cell strains that have never been stored at −70°C, including their diploid karyotype.

Susceptibility of diploid human cell strains to viruses.—The WI-1 and WI-10 strains of diploid human fibroblasts were used, for the most part, in determining virus susceptibility. The virus titrations were made from the 17th to the 35th subcultivation of these cells. The qualitative results are tabulated in Table III. Tubes were prepared each with 5×10^4 cells per ml in GM. The tubes were refed 24 hr after planting and titrations were performed using three tubes for each tenfold dilution of virus. The final titrations yielded values consistently less than those obtained with the system in which the virus pool had originally been grown. WI-1 cells reconstituted from the frozen state did not vary in virus susceptibility. The end points of titrations made in WI-1 cells were read on the 6th day post inoculation. Subsequent production of pools of Polio Strain Koprowski-Chat in strain WI-1 yielded

[1] Assuming a 2:1 split ratio, the theoretical maximum cell yield for 50 generations is 10^{22} cells where 1×10^7 cells can be obtained from one Blake bottle. This total potential yield is equal to 2×10^7 metric tons of cells based on 5×10^8 cells equivalent to 1 g wet weight.

TABLE III. *Virus susceptibility of human diploid cell strains.*

Cell strain	Virus	Cytopathogenic effect
	Measles	+
	COE	+
	Adenovirus Type 2	+
	Adenovirus Type 12	+
	Coxsackie A 9[a]	+
	Coxsackie A 13	+
	ECHO 9	+
	Reovirus Type 1[a]	+
	Poliovirus Koprowski–Chat Type 1 (attenuated)	+
	Poliovirus Strain TN Type 2 (attenuated)	+
WI-1	Poliovirus Koprowski–Fox Type 3 (attenuated)	+
(Passages	Poliovirus Type 1 (Mahoney)	+
20–35)	ECHO 21	+
	Varicella[b]	+
	Rabies Strain CVS	+ (see text)
	Mengo	+
	Endomyocarditis virus (EMC)	+
	Herpes simplex	+
	Vaccinia	+
	Yellow Fever, Strain 17D	+
	Influenza Type A, Strain jap	+
	Influenza Type B, Strain lee	+
	Influenza Type C, Strain colindale	−
	Coxsackie B 1	−
	Coxsackie A 1	−
	Western Equine Encephalitis	−
	Polyoma	−
	ECHO 11[a]	+
	ECHO 20[a]	+
	ECHO 22[a]	+
	ECHO 28[a]	+
	Respiratory Syncytial (Strain Long)[a]	+
	HE virus (Pett. strain)[a]	+
WI-10	Salisbury strain H.G.P.[a]	+
(Passages	Salisbury strain F.E.B.[a]	+
17–25)	Parainfluenza 1[a, c]	−
	Parainfluenza 2[a, c]	−
	Parainfluenza 3[a, c]	+

[a] Tested by Drs. Vincent V. Hamparian, Albert Ketler, and Maurice R. Hilleman of the Division of Virus and Tissue Culture Research, Merck Institute for Therapeutic Research, West Point, Pa. [b] Tested by Dr. Eugene Rosanoff, Wyeth Laboratories, Radnor, Pa.
[c] Detected by hemadsorption.

titers of $10^{-7.2}$, indicating that with the passage of other viruses in the diploid strains, higher titers may be achieved. WI-1 cell degeneration in the presence of Polio Strain Koprowski-Chat is illustrated in Fig. 18.

The growth of the CVS 24 strain of rabies fixed virus in the WI-1 strain was determined by mouse inoculations, since a variable cytopathogenic effect was observed. A complete cytopathogenic effect was observed with most of the viruses listed in Table III. A culture of WI-1 cells inoculated with this strain of rabies virus continued to replicate virus for periods up to one month after periodic complete medium changes as measured *in vivo*. The cultures continued to metabolize during this time. When the medium became acid every 4–5 days, the sheet was washed with PBS and refed. Intracerebral inoculations of 3 to 4-week-old Swiss mice with aliquots of spent medium taken 1 and 4 weeks post inoculation of the WI-1 culture and fluorescent antibody staining of cell sheets at $2\frac{1}{2}$ weeks showed the presence of rabies virus [25, 26]. Recent reports [10, 27] indicate similar growth of rabies virus in primary hamster kidney cells with no concomitant cytopathology. The experiments with this virus in WI-1 cells indicate that rabies virus can now be grown in nonneural human cells *in vitro*.

Plaque formation was also readily obtained with WI-1 cells inoculated with Koprowski-Chat poliovirus Type 1 as indicated in Fig. 19 and with Poliovirus strain Mahoney as indicated in Fig. 20. Characteristically smaller plaques were obtained with Chat than with Mahoney.

It is also of interest that Coxsackie A9 can be grown in passaged WI-1 cells since it has been reported that this virus can only be grown on primary primate cells [32]. The Salisbury strains [60], which are closely identified with the common cold, were also observed to give an unmistakable cytopathogenic effect in high passaged human kidney and lung strains. Varicella reacted similarly in high passaged human lung strains.

Implantation of diploid human cell strains into hamster cheek pouches.— Since it is known that heteroploid cell lines of malignant origin will form tumors which develop progressively when implanted into the hamster cheek

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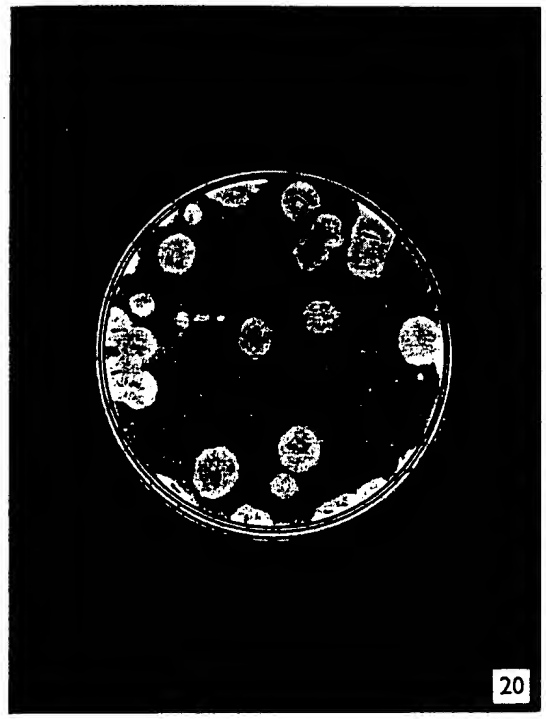
Fig. 20.—Strain WI-1, 30th passage. Plaque formation in the presence of Poliovirus Type 1, strain Mahoney. 50 mm Petri dish.

Fig. 21.—Strain WI-1, 35th passage. Multilayer growth after prolonged incubation. Each cell plane is oriented in a different direction. May-Grünwald Giemsa stain. ×140.

Fig. 22.—Strain WI-1, 35th passage. Incubation for one month in a 50 mm Petri dish. Note membrane is beginning to curl away from the edges of the dish. May-Grünwald Giemsa stain.

Fig. 23.—Strain WI-1, 39th passage. Membrane produced in a Blake bottle and curled up in the medium. ×12.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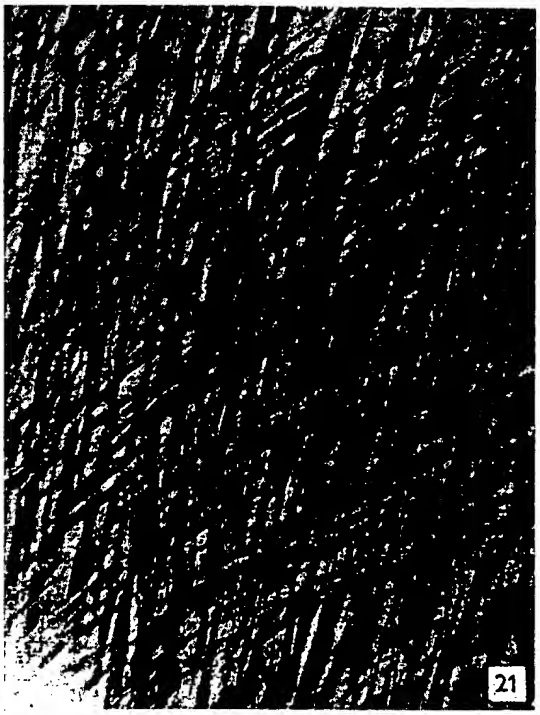
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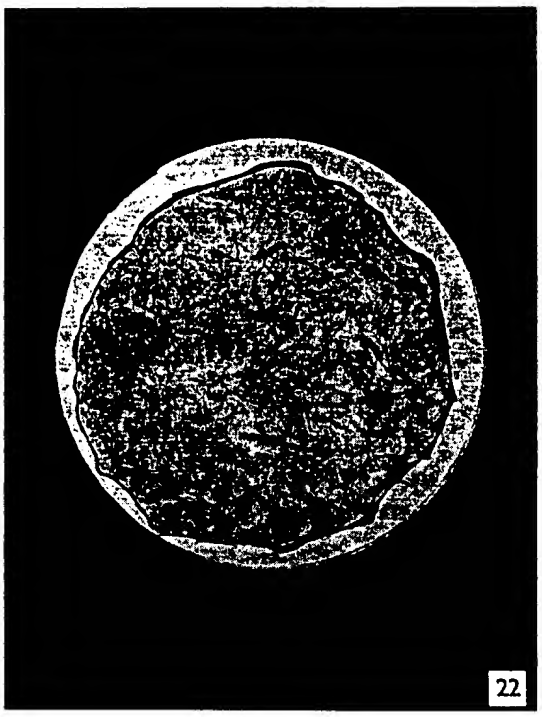
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pouch [12, 13, 15], the degree of heterotransplantability of human diploid cell strains was investigated. After 7 days, three of the five hamsters receiving 10^6 WI-25 cells in the 14th passage showed small nodules. Animals receiving less than this amount never revealed nodules. The 5 hamsters receiving 10^5 HeLa cells all showed nodule formation. After 14 days the nodules in three of the five animals receiving 10^6 WI-25 cells had regressed considerably, while those receiving HeLa cells had enlarged. After 21 days only one very small nodule remained in one hamster receiving the WI-25 cells. This nodule was biopsied and regarded as a hemorrhagic area with an inflammatory response. The nodules of two hamsters receiving HeLa cells were also biopsied at this time and it was determined that there was progressive cellular growth with vascularization.

As pointed out by Foley and Handler [13], cell lines derived from neoplastic tissue are frankly invasive and exhibit a greater growth potential than those derived from non-malignant tissue when titrated in unconditioned hamsters. Accordingly all cell lines of malignant origin examined by these investigators formed tumors in cell concentrations of 10^6 per hamster. Lower cell concentrations and the fate of the nodule served to distinguish between cell lines originating from malignant tissue and those from normal tissue. It is therefore of interest that the diploid human cell strain WI-25, which was inoculated in concentrations as high as 10^6 cells per animal, caused no tumor formation, and it is concluded that even after 14 passages *in vitro* strain WI-25 had not acquired the degree of malignancy associated with those cell lines investigated by Foley and Handler.

Homotransplantation of WI-1 cells into terminal cancer patients.—The WI-1 strain was selected for studies paralleling those performed by Southam, Moore and Rhoads [56]. These workers showed that when cells from heteroploid human cell lines (derived from both normal and malignant tissue) were inoculated into terminal cancer patients, they multiplied in most of the recipients, as indicated by the formation of a palpable nodule at the site of implantation; and that upon biopsy healthy cancer cells with active mitosis were found. The only "normal" cells utilized by these investigators were human embryonic fibroblasts with "normal cytology". In this case examination of recipient cancer patients revealed that no growth took place at the site of inoculation but that cell line cells inoculated simultaneously into the same patients did grow.

It is presumed that the five preparations of normal human embryonic fibroblasts used by these workers were primary cells and therefore diploid [36, 56]. Our objective was to determine whether WI-1 cells that had been

subcultivated up to 45 times *in vitro*, and that appeared to be normal by all other criteria, would react in the same way as the primary fetal fibroblasts of Southam *et al.* [56] and unlike their heteroploid cell lines.

The results of this study are shown in Table IV. As pointed out by Southam *et al.* [56]: "Slight local induration and erythema frequently followed inoculations but subsided completely by the third day." Although our results indicated that nodules could be found in some cases up to the seventh day, biopsies of these nodules did not reveal anything suggestive of the results

TABLE IV. *Results of homotransplantation of WI-1 cells into terminal cancer patients.*

Patient	Diagnosis	Therapy	Pool inoculated	Results	Biopsy results
A.L.	Diffuse abdominal carcinomatosis	Symptomatic	1	Nodule developed and disappeared on 6th day.	Not done.
M.G.	Metastatic breast carcinoma	Prednisone for 8 weeks prior to and after homotransplantation.	1	5 mm nodule formed on 6th day. Disappeared by 10th day. Patient died on 14th day.	Not done.
W.H.	Metastatic bronchogenic carcinoma	Pronounced leukopenia from chemotherapy (nitrogen mustard)	2	3 mm nodule formed on 6th day. Biopsied.	Negative
R.M.	Metastatic carcinoma of the colon	5-fluorouracil given 1 week prior to homotransplatation (no leukopenia)	2	No nodule developed up to 9 days.	Not done.
S.J.	Metastatic breast carcinoma	Pancytopenia following Cytoxan given 2 days before homotransplantation.	2	Minimum induration after 7 days. Biopsied. Patient died on 8th day.	Negative
P.M.	Metastatic breast carcinoma	Mild leukopenia following Cytoxan administration 9 days prior to homotransplantation.	2	No nodule developed. Biopsied on 7th day.	Negative

obtained by Southam *et al.* [56] when they inoculated heteroploid cell lines. None of the biopsies revealed anything of an abnormal character and the presence or growth of the inoculated WI-1 cells could not be detected. In two of our patients (A.L. and M.G.) where nodules developed, subsequent total regression was observed. In two other patients (W.H. and S.J.) where nodules developed and were biopsied, nothing abnormal was found. The two remaining patients (R.M. and P.M.) developed no nodules.

These results indicate that the WI-1 strain of embryonic, diploid, human fibroblasts subcultivated up to 45 passages does not produce the kind of growth obtained when heteroploid cell lines are inoculated into human cancer patients. The activity of the WI-1 cells closely parallels the results obtained when inoculating primary human fetal cells under similar conditions. It is therefore concluded that although the WI-1 strain was propagated for 45 passages over a period of 10 months, with intervening storage at −70°C, the malignant characteristics exhibited by heteroploid cell lines were not acquired as demonstrated by inoculation into terminal cancer patients.

Histotypic growth of human diploid cell strains.—When a confluent culture of any diploid cell strain is kept at 36°C for approximately one month, with 1 or 2 pH adjustments, it is found that in almost all cases the cell sheet formed is not a monolayer of fibroblasts, but is instead a multilayer cell sheet, or membrane. The membrane formed is such that each layer of fibroblasts is oriented in a different direction as illustrated in Fig. 21 for the WI-1 strain. After about one month the entire membrane will start to peel from the glass surface at the edges (Fig. 22) and can often be found floating free in the medium. Agitation of the culture vessel will cause the membrane to roll up as illustrated in Fig. 23. Slight agitation of the culture vessel may help to lift the membrane from the glass surface and such membranes can be spread out on a glass slide, stained, and examined microscopically (Fig. 24). The rolled up membranes can also be fixed, imbedded, sectioned, and stained using routine histological methods.

Hemotoxylin and eosin stains of a representative number of such membranes were performed, some of which are illustrated in Figs. 25 and 26. They illustrate the appearance of a strain WI-1 membrane from the 36th

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Fig. 24.—Strain WI-1, 36th passage. Membrane spread out on the surface of a glass slide, fixed and stained with May-Grünwald Giemsa. ×1.

Fig. 25.—Strain WI-1, 36th passage. Microscopic appearance of a membrane. Hematoxylin-eosin. Note multilayer cellular organization and intercellular matrix. ×90.

Fig. 26.—Same as Fig. 25. Higher power magnification. ×410.

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subcultivation representing a period of time in cultivation of 7 months. Many of the membranes that were detached from glass were fixed in Tellyesniezky's fixative, imbedded in paraffin and sectioned as routine histological material. Consecutive slides were stained with hematoxylin–eosin, Weigert–Van Gieson for the identification of elastic and connective tissue, and Rocque's chromotrope and toluidine blue with alcohol differentiation. At microscopic examination the membranes appeared to be constituted of an inner portion of amorphous eosinophilic material, lined on both sides by one or two cell layers. The cells were essentially round and occasionally a cell or group of cells of cylindrical shape had one border attached to the membrane. The amorphous material stained a pale red with the Van Gieson, blue with the chromotrope, and did not show any metachromasia with the toluidine blue. No fibrillar structure was observed with light microscopy. Several groups of pyknotic nuclei at different stages of degeneration were observed in some fields. Similar membrane formation with the same histological characteristics were observed with WI-8, WI-9 and WI-23 strains. The amorphous matrix does not contain acid mucopolysaccharide and the staining reactions seem to suggest the presence of collagen. The ultrastructure of this matrix is now being studied with the electron microscope.

The results are compatible with an interpretation of primitive histotypical differentiation characterized by the ability of the cells to lay down an intercellular matrix and to organize into a multilayered membrane, suggesting a retention of some functional capacities.

DISCUSSION

The experiments herein described illustrate the simplicity with which strains of human fibroblasts can be kept in serial cultivation for long periods of time with maintenance of the integrity of the diploid karyotype. It is apparent that the exacting conditions described by others [41, 47] are not critical in order to achieve this result.

Chromosome cytology and sex chromatin.—The observed conformity between sex chromatin characterization and chromosomal sex exhibited through at least 44 passages fulfills expectations for primary cells in culture [34, 35]. Others report reduction or loss of sex chromatin with continued subcultivations [43] and of variations in number per nucleus in cell lines [6].

The absence of evidence of heteroploid alteration in the 13 strains studied implies a "stability" of the diploid cell in culture in spite of repeated trypsinization which is known to increase division anomalies in mouse cells [31].

However, since alterations to heteroploidy occur haphazardly and under influences poorly understood, chromosome monitoring is necessary to establish confidence in any particular strain. While chromosome counts alone may rule out heteroploidy and aneuploidy, monitoring must be extended to include karyotype analysis for those changes which are less obvious, such as quasi-diploidy [14].

Although certain human chromosomes are specifically identifiable, some rearrangements between and within those chromosomes recognizable only as members of a group would probably pass unnoticed. Such "cryptostructural" changes in relation to cancer hypotheses have been stressed by Hauschka and Levan [16]. An appreciation of the limits of detection of visible chromosome aberrations is afforded by the recent finding made by Nowell and Hungerford [42], and independently by others [2]. These workers found a chromosomal change in blood cells consistently associated with chronic granulocytic leukemia which involved a reduction in length of one of the smallest chromosomes comprising less than 0.8 per cent of the total haploid chromosome length. Aside from "cryptostructural" limitations, it should be stated that however large the sample of the dividing population, absolute certainty as to the exclusion of chromosomally altered cells is impossible. However, considerations concerning "overgrowth" or replacement of the original cell population by the altered cell and sampling at various subculture levels should ensure detection of heteroploid alterations.

Obtaining nearly limitless numbers of cells of a uniform genetic constitution has marked practical advantages over using the pleiomorphic population comprising the cell line. This is analogous to the use of inbred as compared with randomly bred animals for biological study. *In vitro* studies of the diploid somatic cell as well as specific types of trisomics, monosomics, etc. should prove valuable. A cell strain has been obtained from a mongol (now in the 25th passage) which remains trisomic for chromosome #21 as is characteristic of the tissue of origin [33].

The Phase III phenomenon.—The characteristic degeneration typical of Phase III in the history of diploid cell strains, wherein the strain loses its growth potential and is itself gradually lost, has important implications. Of three possible explanations of this phenomenon that are considered, only one appears to be acceptable.

The fact that Phase III occurs in one strain of cells while other strains from the same or from different fetuses are luxuriating in Phase II in the same medium pool apparently excludes lack of metabolites or presence of toxic materials in the medium as an explanation for this phenomenon.

Furthermore, if cells from Phase II are restored from the frozen state in the same pool of medium that is being used on the continuous passage cells of that strain in Phase III, luxurious growth invariably results. It appears, therefore, that the growth medium used for these experiments is entirely adequate; and inasmuch as about 15 separate pools of calf serum have been employed throughout this work the impression is that no "toxic" sera were encountered. No attempt was made to pretest the medium. Swim and Parker [57] arrived at a similar conclusion, with cell strains assumed by us to be diploid.

A second explanation for the eventual loss of the diploid strains, or their entry into Phase III, is the possibility that some pool of essential metabolites originally present in the primary cell population and not synthesized during *in vitro* cultivation is gradually depleted. In the case of the WI-1 strain, such a possibility would require a hypothetical pool originally present in the cell population large enough to impart at least one molecule to those cells of the 50th passage. This possibility can be rejected on purely mathematical grounds.

The 50th passage represents more than 2^{50} generations or 10^{15} progeny. Each cell isolated from the fetal lung tissue must then contain a pool of the hypothetical metabolite in a concentration of 10^{15} molecules, assuming equal distribution to daughter cells. A pool of 10^{15} hydrogen molecules would weight 3.3×10^{-9} g. Since one WI-1 cell weighs approximately 2×10^{-9} g (based on 5×10^{8} cells equivalent to 1 g wet weight) this hypothesis is invalid.

The third possible explanation for entry into Phase III may bear directly upon problems of ageing, or more precisely, "senescence". This concept, although vague at the level of the whole organism, may have some validity in explaining the phenomenon at the cellular level, at least as an operational concept.

This may be explained by postulating a factor, necessary for cell survival, whose rate of duplication is less than that of the cell (asynchronous). It is possible to conceive of two separate self-duplicating systems one of which (the hypothetical) is contained within the other (the cell). A slight reduction in replication rate of the hypothetical system could possibly lead to a gradual depletion of this factor to a critical or threshold level within 40–50 generations. Conversely the rate of synthesis itself may be unchanged, but a slightly higher rate of loss (through some unknown *in vitro* condition) would eventually yield the same result. This may be interpreted as a cumulative effect.

A parallel situation exists in biology in a number of protozoa and has been well investigated in certain species of Paramecium which contain the Kappa factor [55]. This factor is a self-duplicating system within the Para-

mecium and multiplies at a rate that can be influenced by the environment independently of the rate of multiplication of the Paramecium. Thus such Kappa containing Paramecium or killer organisms can be depleted of their Kappa by manipulating environmental conditions in such a way that the Paramecium can "outgrow" the Kappa factor and become a non-killer. As pointed out by Sonneborn [55]: "The maintenance of Kappa appears to be precarious. It does not necessarily divide synchronously with the Paramecia. It is distributed, not precisely, but randomly to the products of fission. It often cannot keep pace with the reproduction of the Paramecia; this can result in total loss which is irreversible."

This concept appears to explain best the fact that in our experiments and those of others [41, 47, 48, 58, 59] no one has succeeded in carrying diploid human cells for periods much over one year. It is not implied that any exact number of passages are required or that a definite period of time is necessary to predispose the cells to Phase III, but simply that there does exist a finite limit to the cultivation period of diploid cell strains (Text-Fig. 1). The fact that heteroploid cell lines can apparently be carried for indefinite periods of time may be compared directly with transplantable tumors. Both can be cultivated indefinitely and both are heteroploid. It is interesting to speculate on the possibility of constructing an analogous situation with the diploid cell strains. Could mammalian diploid strains having an appropriate marker (e.g. sex chromatin) be carried indefinitely *in vivo* in isologous hosts which would indicate an escape by the marked cells from the phenomenon of senescence?

Although no abnormalities were noted in the dividing population just prior to Phase III, the bizarre interphase nuclei seen may represent mitotically incompetent cells. Sax and Passano [54] have shown that "culture age is a major factor in the incidence of spontaneous chromatid aberrations" seen at anaphase.

Histotypical differentiation.—Diploid human strains cultivated under the conditions described form membranes consisting of a multilayer of cells with the concomitant formation of an interstitial matrix. This membrane formation has been observed, when last attempted, as late as the 36th subcultivation (7 months *in vitro*) with the WI-1 strain. It has long been felt by organ culturists that in order to retain tissue organization *in vitro* conditions must be provided other than those required for rapid multiplication. By adjusting the pH and thus depriving the cells of any new source of nutrients (glucose?) which are presumed to be exhausted, membrane formation has been achieved in these diploid strains.

The fact that only a very primitive type of membrane develops may be related to the observation that all of the human strains studied in this way were composed exclusively of fibroblasts (beyond the 5th passage) without detection of any epithelial elements. The use of such membranes for wound repair is under investigation. It may be surmised that the type of histotypical development observed is restricted to that type of organization possible with stromal elements only. It may be postulated, therefore, that if methods could be developed to retain all types of cells present in the primary tissue, a greater degree of differentiation could be obtained. Inclusion of all cellular elements, which is apparently the case when reaggregation of primary cells occurs *in vitro*, is demonstrable when no serial cultivations intervene. This results in the histotypical differentiation reported by Moscona [40] and the more spectacular morphological organization reported by Weiss [61] when dissociated chick embryo cells are grown on the chorioallantoic membrane of 8-day chick embryos. It is important, therefore, that human diploid fibroblasts can, after repeated subcultivations, exposures to trypsin, and passages of considerable lengths of time, form membranes with some degree of histotypical differentiation. Such differentiation, primitive as it is, serves to underline the concepts of Weiss [61] which stress the role of internal "self organization" as opposed to the more classic views, which emphasize the role of "inducers" in supplying cellular "information" from without.

Virus sensitivity of diploid human cell strains.—The cytopathogenic effect observed when a broad spectrum of viruses was titrated on the WI-1 and WI-10 strains indicates the high degree of susceptibility of this type of cell to virus infection. The variations in diploid human cell strain susceptibility to the Koprowski-Chat Type 1 strain of poliovirus had more of a quantitative than a qualitative difference (Table V). It is our impression that the consistently lower titers of all viruses titrated in the WI-1 strain as opposed to titers obtained in the optimum cell system may be a result of titration in the WI-1 strain directly from virus pools grown in the optimum system. Where the Koprowski-Chat Type 1 poliovirus was first grown in the WI-1 cells and then titrated in these cells, the titer obtained was *not* appreciably lower than titrations made in primary monkey kidney (Table V).

This raises the question of the use of diploid human cell strains for the production of killed or attenuated human virus vaccines; and in particular poliovirus vaccines. The objections raised against using heteroploid cell lines in the production of human virus vaccines have been pointed out by Westwood [62]: "It is the fear of malignancy more than any other single factor which precludes the cell lines at present available from use in the

production of virus vaccines." In view of the filtration procedures used in making oral polio vaccines the question of feeding live cells can be discounted. As pointed out further by Westwood [62]: "The risk lies in the possibility of inducing malignant changes in the cells of the human subject by the introduction of an, as yet hypothetical, virus or non-living transforming principle analogous to that inducing change of type in the pneumococcus."

TABLE V. *Comparative titrations of poliovirus Koprowski-Chat Type 1 in diploid human cell strains.*

Cells	No. of passages	$TCID_{50}$/ml.
Monkey kidney	Primary	$10^{-7.5}$
HeLa	Cell line	$10^{-6.5}$
WI-1[a]	35	$10^{-7.2}$
WI-13	26	$10^{-6.5}$
WI-5	27	$10^{-6.5}$
WI-9	24	$10^{-5.7}$
WI-15	23	$10^{-5.7}$
WI-11	23	$10^{-5.5}$
WI-14	30	$10^{-3.0}$
WI-12	32	$10^{-3.0}$

[a] Virus pool inoculated, previously grown in WI-1. All other cells titrated with a virus pool grown in primary monkey kidney.

In view of these objections human poliovirus vaccines (attenuated and killed) are now acceptable in this country only when grown in primary monkey kidney, as such tissue is presumed to have no malignant properties.[1]

Serious objections, however, may be raised even to the use of this tissue. It is well known that primary monkey kidney has a very high content of latent simian viruses. Indeed, at least 18 such viruses have now been isolated from primary monkey kidney [24]. One of these latent viruses (B virus) is even known to have caused fatalities in man [45]. It is now becoming apparent that all primary monkey kidney may contain one or more latent viruses whose characteristic cytopathology becomes evident when such tissue is cultured *in vitro*. The appearance of such viruses and the associated cytopathology is probably a reasonable explanation of the fact that such

[1] *Note added in proof.*—A recent report (Eddy, B. E. *et al.*, *Proc. Soc. Exptl. Biol. Med.* **107**, 191 (1961)) indicates that extracts of pools of monkey kidney cells are capable of inducing neoplasms in hamsters. Krooth, R. S. and Tjio, J. H. (*Virology* **14**, 289 (1961)) have independently reported on the growth of poliovirus strain mahoney in human diploid cell strains.

tissue cannot be subcultured successfully beyond about the 5th passage. This excludes those rare cases [44] in which alterations to heteroploidy occurred. This degenerative phenomenon has also been observed when certain members of the adenovirus group are unmasked in tonsil and adenoid tissue cultivated *in vitro* [52, 53].

The isolation and characterization of human diploid cell strains from fetal tissue make this type of cell available as a substrate for the production of live virus vaccines. Other than the economical advantages, such strains, in contrast to the heteroploid cell lines, exhibit those characteristics usually reserved for "normal" or "primary" cells (Table IV) and therefore make the consideration of their use in the production of human virus vaccines a distinct possibility.

TABLE VI. *Differential characteristics for human cell lines and cell strains.*

Character	Cell lines	Cell strains
1. Chromosome number	Heteroploid	Diploid
2. Sex chromatin	Not retained or variable	Retained
3. Histotypical differentiation	Not retained	Partially retained
4. Growth in suspended culture	Generally successful	Unsuccessful
5. Pathological criteria for malignancy as determined on biopsies of cells inoculated into hamsters or human terminal cancer patients	Positive	Negative
6. Limitation of cell multiplication (life of strain or line)	Unlimited	Limited
7. Virus spectrum compared to corresponding primary tissue	Often different	Same
8. Cell morphology compared to corresponding primary tissue	Characteristically different	Same
9. Acid production	Less than that produced by equal number of cell strain cells	More than that produced by equal number of cell line cells
10. Retention of Coxsackie A 9 receptor substance	Lost	Retained
11. Ease of establishment	Difficult (not predictable)	Usually successful

The question of the presence of latent viruses in any cellular material is one that can probably never be answered with absolute certainty; yet it is possible to perform exhaustive studies with techniques now available (e.g. irradiation) to rule out effectively the presence of latent viruses in one strain of diploid cells so that attention can be concentrated on the use of such a "clean" strain for the production of live human virus vaccines.

It would not be necessary to test large numbers of such strains for latent virus content. Even though these strains do degenerate as late as the 50th passage (strain WI-1), if all the surplus cells from each subcultivation were stored in the frozen state a potential yield of 20 metric tons of cells could be obtained from any single strain if its "passage potential" was even as low as 30 subcultivations. Clearly, the potential "senescence" of any diploid strain should not detract from its usefulness, since the potential cell yield is abundant, if not inexhaustible, for all practical purposes.

SUMMARY

The isolation and characterization of 25 strains of human diploid fibroblasts derived from fetuses are described. Routine tissue culture techniques were employed. Other than maintenance of the diploid karyotype, ten other criteria serve to distinguish these strains from heteroploid cell lines. These include retention of sex chromatin, histotypical differentiation, inadaptability to suspended culture, non-malignant characteristics *in vivo*, finite limit of cultivation, similar virus spectrum to primary tissue, similar cell morphology to primary tissue, increased acid production compared to cell lines, retention of Coxsackie A9 receptor substance, and ease with which strains can be developed.

Survival of cell strains at −70°C with retention of all characteristics insures an almost unlimited supply of any strain regardless of the fact that they degenerate after about 50 subcultivations and one year in culture. A consideration of the cause of the eventual degeneration of these strains leads to the hypothesis that non-cumulative external factors are excluded and that the phenomenon is attributable to intrinsic factors which are expressed as senescence at the cellular level.

With these characteristics and their extremely broad virus spectrum, the use of diploid human cell strains for human virus vaccine production is suggested. In view of these observations a number of terms used by cell culturists are redefined.

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